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Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering ☆

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Abstract

Objective: In tissue engineering, three-dimensional biodegradable scaffolds are generally used as a basic structure for cell anchorage, cell proliferation and cell differentiation. The currently used biodegradable scaffolds in cardiovascular tissue engineering are potentially immunogenic, they show toxic degradation and inflammatory reactions. The aim of this study is to establish a new three-dimensional cell culture system within cells achieve uniform distribution and quick tissue development and with no toxic degradation or inflammatory reactions. **Methods**: Human aortic tissue is harvested from the ascending aorta in the operation room and worked up to pure human myofibroblasts cultures. These human myofibroblasts cultures are suspended in fibrinogen solution and seeded into 6-well culture plates for cell development for 4 weeks and supplemented with different concentrations of aprotinin. Hydroxyproline assay and histological studies were performed to evaluate the tissue development in these fibrin gel structures. **Results**: The light microscopy and the transmission electron microscopy studies for tissue development based on the three-dimensional fibrin gel structures showed homogenous cell growth and confluent collagen production. No toxic degradation or inflammatory reactions could be detected. Furthermore, fibrin gel myofibroblasts structures dissolved within 2 days in medium without aprotinin, but medium supplemented with higher concentration of aprotinin retained the three-dimensional structure and had a higher collagen content ($P < 0.005$) and a better tissue development. **Conclusions**: A three-dimensional fibrin gel structure can serve as a useful scaffold for tissue engineering with controlled degradation, excellent seeding effects and good tissue development.

Keywords: Tissue engineering; Scaffold; Fibrin gel; Cardiovascular

1. Introduction

Heart valve replacements are routinely performed cardiac operations for treating severely diseased valves. Commonly used valve substitutes include mechanical valves, bioprosthetic valves and homografts. These prosthetic valves function well, but each of them has its own inherent limitations. This leads to a series of studies to determine, if tissue engineering principles can be used to develop valve tissue substitutes out of isolated autologous cells to avoid the known limitations of the common heart valve prosthesis [1–4].

The most common mode of engineering heart valve tissue is based on using a three-dimensional biodegradable scaffold for cell anchorage. The scaffold serves as a synthetic template for cell growth and tissue development during in vitro culture. First, cells have to be seeded on the scaffold and then the cells are going to attach the fibers for further proliferation and differentiation. The scaffold degrades while tissue development [5].

Ideally, scaffolds for tissue engineering should provide a high surface area for cell-polymer interactions, sufficient space for extracellular matrix regeneration, and minimal diffusional constraints during in vitro culture. The scaffolds should resorb once it has served its purpose of providing a template for regeneration tissue, and the scaffold degradation rate should be adjustable to match the rate of tissue regeneration [6]. The currently used materials for maintaining three-dimensional structures in tissue engineering are either polymers composed of chemical substances like polyglycolic acid or polyhydroxybutyrate or gels out of extracellular matrix proteins such as collagen. However, these materials are still far from ideal, they are expensive and

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potentially immunogenic and in addition they show toxic degradation and inflammatory reactions [6–8].

Fibrin gel can be produced of the patients blood and be used as an autologous scaffold for the seeded fibroblasts to create a three-dimensional structure and furthermore no toxic degradation or inflammatory reactions will be expected.

2. Material and methods

2.1. Human aortic myofibroblast cell expansion

Human aortic tissue was harvested from the ascending aorta in the operating room. After harvesting, the explants were rinsed with phosphate-buffered saline (PBS) and stripped off adventitia with scissors under a laminar flow hood. The tissue was then cut into small pieces of 2×2 mm for primary culture in 75 cm^2 vented polystyrene cell culture flasks (Falcon 3111, Becton Dickinson, Lincoln Park, NJ) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% streptomycin (Gibco BRL–Life Technologies, Grand Island, NY). After 3–4 weeks, human aortic myofibroblasts grew into confluent monolayers and were serially passaged by trypsinization (trypsin/EDTA solution, 0.05/0.02%, Gibco BRL-Life Technologies, 3–5 min) and subcultured to obtain sufficient cell numbers for cell seeding (passage 3–4).

2.2. Preparation of fibrin gel

Fifty milligrams plasminogen-free fibrinogen from pooled human plasma (Fluka) was dissolved in 3 ml distilled water and dialyzed vs. Tris-buffered saline (TBS) overnight at room temperature. The solution was filtered through a 5 μm and 0.22 μm filter for sterilization. The fibrinogen concentration was determined by measuring the absorbance at 280 nm. The volume required to obtain 3.5 mg of fibrinogen in the seeding was calculated based on the determined fibrinogen concentrations.

2.3. Seeding

Seedings were performed in polystyrene 6-well flat-bottom culture plates (Costar 3516, Cambridge, MA). To construct a 1-mm thick cell-fibrin gel structure a 6-well culture plate, 750 000 human myofibroblasts were suspended in 1 ml fibrinogen solution composed of 50 mM $CaCl_2$ 50 μl, 100 μl thrombin (20 U/ml in TBS)(Sigma Inc.), 350 μl TBS, 500 μl fibrinogen. The final fibrin gels contains 3.5 mg fibrinogen, 2.5 mM Ca^{++}, and 2 NIH units thrombin. The culture plates were then put in cell culture incubator for polymerization for 1 h. Afterwards 5 ml medium was added to the seeded plates.

2.4. Culture

The cell-fibrin gel structure was cultured for 1 month with Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% streptomycin (Gibco BRL–Life Technologies). The medium was further supplemented with 1.0 mM L-ascorbic acid 2-phosphate, 0.4 M proline (Sigma Chemical Co, St. Louis, MO) and different concentrations of aprotinin (5, 10 and 20 μg/ml) and was changed on a daily base.

2.5. Structure assessment

2.5.1. Collagen content

Collagen content was estimated by hydroxyproline assay according to Reddy's method [9]. Briefly, samples were lyophilized and hydrolyzed with 2 M NaOH. Hydrolyzed free hydroxyproline was oxidized with chloramine-T and the addition of Ehrlich's reagent resulted in the formation of a chromophore that was measured at 550 nm.

2.5.2. Histological evaluation

Specimens for light microscope examination were fixed in 4% formalin, embedded in paraffin and sectioned. The sections were stained by haematoxylin and eosin (HE) or Masson's trichrome. Transmission electron microscopy specimens were fixed with Na-Cacodylat-buffered glutaraldehade (2.5%) and paraformaldehyde (0.8%) solution, and were post-fixed with 1% osmium tetroxide, dehydrated in a series of alcohol, and embedded. Ultra-thin sections were stained with uranyl acetate and lead citrate. The specimens were observed under a transmission electron microscope.

2.5.3. Statistics

Results data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed by ANOVA test. Statistical significance was set at $P < 0.05$. Linear regression analysis was utilized to evaluate the correlation. Data and graphs were proceeded with StatView 4.5 (Abacus Concepts, Inc, Berkeley, CA).

3. Results

The light microscopy and the transmission electron microscopy studies for tissue development based on the three-dimensional fibrin gel structures showed homogenous cell growth and collagen production. The thickness of the tissue was reduced from 1 to 0.8 mm during the culturing period. The tissue showed a uniform cell distribution with a mean cell density of 34 ± 5.7 cells/field. No toxic degradation reactions could be detected (Figs. 1 and 2). In the group with no aprotinin the fibrin gel was dissolved within 2 days. In the group with low aprotinin concentration (5 μg/ml) the gel was phagozyted by the cells after 1 week and the structure appeared to be a thin cellular layer (Fig. 1A). A certain fibrinolysis appeared in the group with 15 μg/ml after 3 weeks but the fibrin gel did not degrade completely. In the group with 20 μg/ml aprotinin no fibrinolysis was visible during the whole culture period. In addition, a three-dimen-

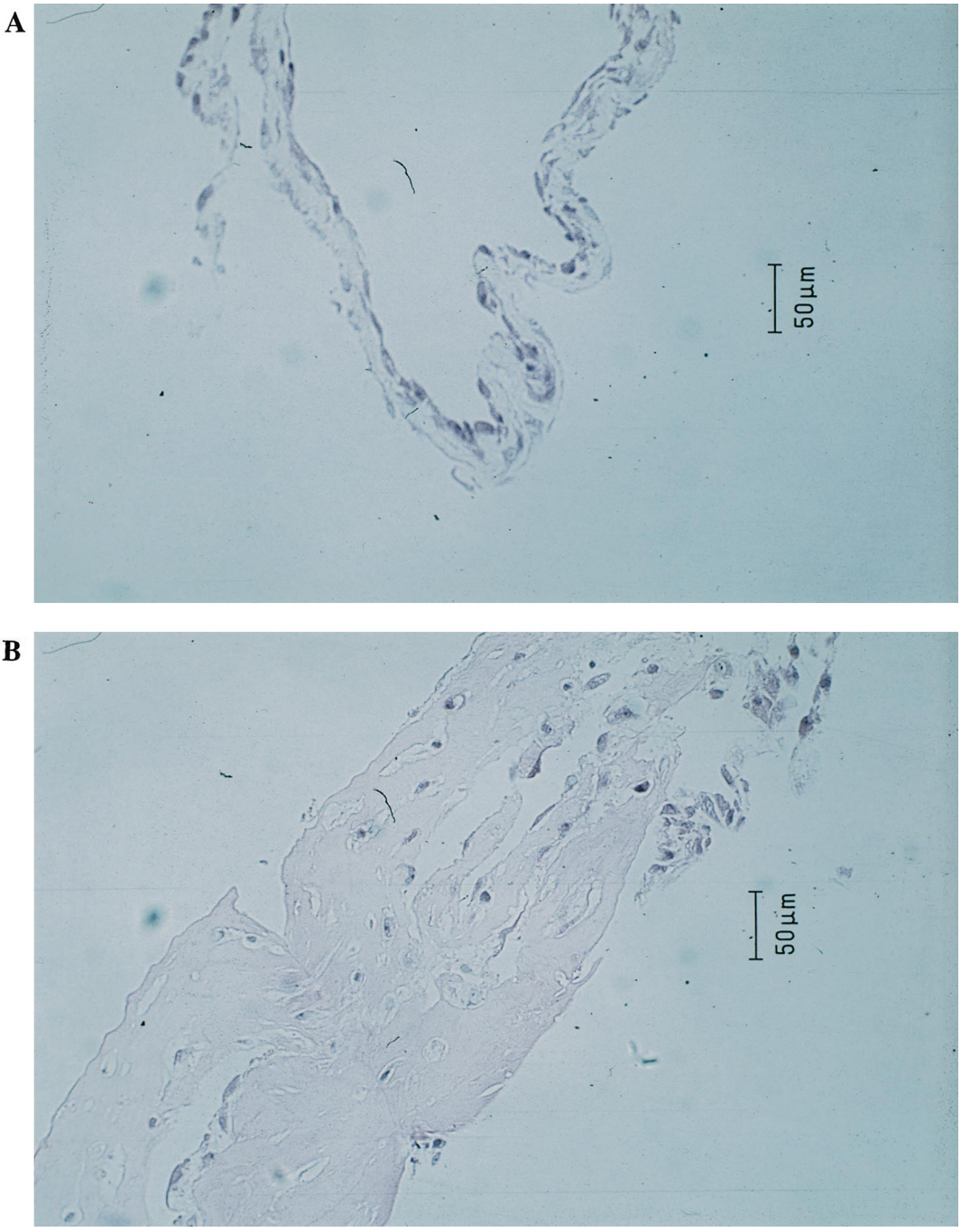

Fig. 1. Light microscopy pictures of cell fibrin gel cultured in different aprotinin concentration. (A) Cultured in lower concentration of aprotinin (5 μg), fibrin gel degraded and the structure appeared to be a thin cellular layer. (B) Cultured in high concentration of aprotinin (20 μg/ml), the cell-fibrin gel structure demonstrated a multilayer-structure with myofibroblasts surrounded by extracellular matrix (hematoxylin and eosin staining).

sional structure with multilayer myofibroblasts surrounded by extracellular matrix was detected in this group (Fig. 1B). Furthermore, the transmission electron microscope documents confluent collagen production and viable cells in the group with high aprotinin concentration (Fig. 2).

After 31 days the hydroxyproline content was measured, which is directly related to the collagen content. A significantly ($P = 0.003$) higher amount of hydroxyproline in the groups with 15 and 20 μg/ml aprotinin compared with the groups with no or low aprotinin concentration was observed (Fig. 3). The biochemical results confirmed the light microscopy and electron microscopy findings.

4. Discussion

Cell distribution and attachment in three dimensions are mainly determined by the gravity [10]. When cells are

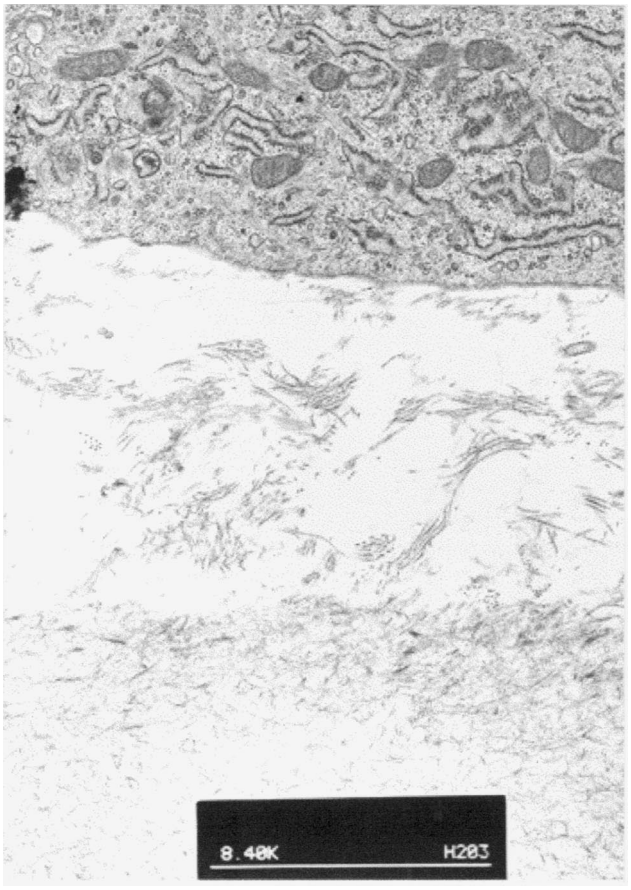

Fig. 2. Transmission electron microscopy documents confluent collagen production and viable cells in the 20 μg/ml aprotinin group and fibrin gel remain undergraded. Scale bar = 3 μm.

seeded onto the scaffolds not all of them will attach. Some will be unattached and are lost for further tissue development. A fast cell attachment on the scaffold provides a spatially uniform distribution of seeded cells. Furthermore, an initial high homogenous cell distribution is associated with high rates of extracellular matrix production [11–13].

Polyglycolic acid polymers (PGA) as scaffolds have been widely studied. They are biodegradable and content a significant surface area for cell attachment. In addition their high porosity guaranties an excellent nutrition supply. Unfortunately the cell attachments on PGA meshes are low and, therefore, they need to be precoated with cell adhesion factors to enhance the cell attachments [14]. Collagen gels as three-dimensional scaffold provides uniform distribution with good cell attachments, but they are not biodegradable and potentially immunogenic. Furthermore there is a big variation between produced collagen batches [15].

In the presented study a three-dimensional fibrin gel structure is used as a scaffold for tissue engineering. Fibrin plays an important role in natural wound healing and is used as sealant and adhesive in surgery. It is formed by the enzymatic polymerization of fibrinogen. A special feature of fibrin, both formed naturally or therapeutically, is that it is degraded and remodelled by cell-associated enzymatic activity during cell migration and wound healing. Aprotinin, a proteinase inhibitor, can slow down or stop fibrinolysis. Aprotinin acts as an inhibitor of human trypsin, plasmin, and plasma and tissue kallikrein by forming reversible-enzyme-inhibitor complexes. It stops fibrinolysis via inhibiting plasmin. In the presented study, different concentrations of aprotinin were used to control the degradation of three dimensional fibrin gel matrix. The results demonstrate, that the degradation of three-dimensional fibrin gel structures is directly adjusted to the supplemented aprotinin concentrations.

In vitro studies recently reported that fibrin gels might have other properties such as promoting cell migration, proliferation, and matrix synthesis through the release of platelet-derived growth factors and the transforming growth factor beta [16]. In addition there is the possibility to incorporate cell mediators like growth factors and other bioactive peptides and proteins into the fibrin gel [17–19]. This would render a more tissue specific environment for the isolated cells and further amelioration of cell function might be expected The gel structure also serves as a semi-permeable membrane, that separates the cells from direct contact with the medium. Collagen and other newly synthesized extracellular matrix components can accumulate in the intercellular space rather than diffusing into surrounding medium. In case of engineering autologous re-implant tissue, the whole procedure might be carried out in an autologous system, in which autologous plasma from the patient itself can be used for producing fibrin gel. The potential possibility of antigenity would completely be eliminated.

In conclusion, a three-dimensional fibrin gel structure can serve as a useful scaffold for tissue engineering with controlled degradation, excellent seeding effects and good tissue development. At that moment the developed tissue with a thickness of 1 mm is not stable enough to create cardiovascular grafts on arterial side. Further investigations

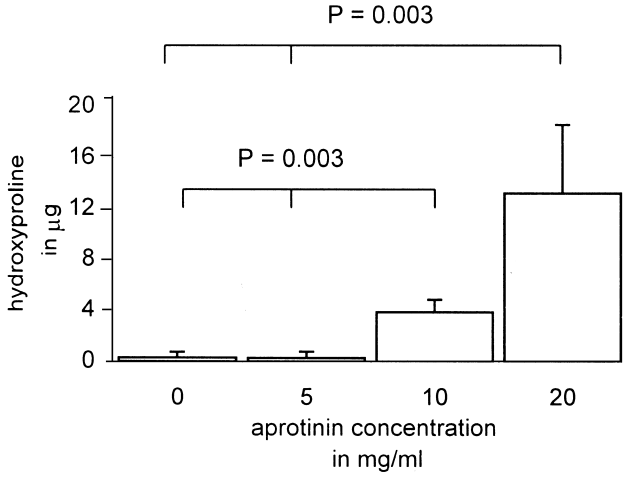

Fig. 3. Comparison of Hyp content in different aprotinin concentration group. More Hyp content was detected in high aprotinin concentration (10 and 20 μg/ml) groups. Hyp = hydroxyproline.

to improve the mechanical properties of the new developed tissue with growth factors, defined mechanical forces are in process. Possible methods of the production of cardiac valves from this tissue are the formation like the production of valves from pericardium. Beyond the formation of cardiovascular structures with the elastic fibrin gel by casting is imaginable in the future.

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